Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress

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Erythropoietin (EPO) promotes neuronal survival after hypoxia and other metabolic insults by largely unknown mechanisms. Apoptosis and necrosis have been proposed as mechanisms of cellular demise, and either could be the target of actions of EPO. This study evaluates whether antiapoptotic mechanisms can account for the neuroprotective actions of EPO. Systemic administration of EPO (5,000 units/kg of body weight, i.p.) after middle-cerebral artery occlusion in rats dramatically reduces the volume of infarction 24 h later, in concert with an almost complete reduction in the number of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling of neurons within the ischemic penumbra. In both pure and mixed neuronal cultures, EPO (0.1–10 units/ml) also inhibits apoptosis induced by serum deprivation or kainic acid exposure. Protection requires pretreatment, consistent with the induction of a gene expression program, and is sustained for 3 days without the continued presence of EPO. EPO (0.3 units/ml) also protects hippocampal neurons against hypoxia-induced neuronal death through activation of extracellular signal-regulated kinases and protein kinase Akt-1/protein kinase B. The action of EPO is not limited to directly promoting cell survival, as EPO is trophic but not mitogenic in cultured neuronal cells. These data suggest that inhibition of neuronal apoptosis underlies short latency protective effects of EPO after cerebral ischemia and other brain injuries. The neurotrophic actions suggest there may be longer-latency effects as well. Evaluation of EPO, a compound established as clinically safe, as neuroprotective therapy in acute brain injury is further supported.

Erythropoietin (EPO) was first characterized as a hematopoietic growth factor (1) and has been in clinical use by millions of patients over the last decade for the treatment of anemia. The observation that EPO and its receptor are expressed in rodent and human brain tissue (2–4), as well as by cultured neurons (5–8) and astrocytes (3, 7, 9), and that EPO has effects on neuronal cells (5), expanded the biological role of EPO beyond hematopoiesis. EPO gene expression in the brain is regulated by hypoxia-inducible factor-1 (1) that is activated by a variety of stressors, including hypoxia. Several independent research groups have reported that EPO protects cultured neurons against glutamate toxicity (6, 10) and reduces ischemic neuronal damage and neurological dysfunction in rodent models of stroke (6, 11–13). We recently reported that systemic administration of EPO is neuroprotective not only in animal models of cerebral ischemia, but also for mechanical trauma, excitotoxins, and neuroinflammation (11). Marked changes in EPO and EPO-receptor (EPOR) gene expression have been reported to occur in brain tissue after ischemic injury (6, 12). Specificity and biological relevance of these changes have been demonstrated by the observation that neutralization of endogenous EPO with soluble EPOR augments ischemic brain damage (13). Thus, it seems that EPO plays a critical role in neuronal survival after hypoxic injury. The nature and mechanism of this protective role, however, are currently unclear.

EPOR belongs to the cytokine receptor type I super family for which substantial information concerning signaling biology exists (14–16). Receptor activation in hematopoietic cells follows after homodimerization on EPO binding, which allows autophosphorylation of EPOR-associated Janus-tyrosine kinase-2 (JAK-2). JAK-2 activation leads to phosphorylation of several downstream signaling pathways, including Ras-mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase [PI(3)K], and the transcription factor Stat5 (signal transducers and activators of transcription; ref. 14). For erythroblasts, the net effect of EPOR stimulation is the inhibition of apoptosis, proliferation, and differentiation (15).

To delineate the nature and mechanism(s) of EPO-induced neuroprotection, we asked whether EPO inhibits neuronal programmed cell death. To accomplish this goal, the effect of EPO on ischemia-induced neuronal DNA damage was first evaluated *in vivo* in a rat model of middle-cerebral artery (MCA) occlusion by using terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL). We then asked whether EPO antagonizes apoptosis induced by serum deprivation, hypoxia, or by application of kainic acid in cultured neural-like P19 teratoma cells and primary rat motoneurons or hippocampal neurons. Does EPO protect neurons through induction of intracellular survival pathways/genes, through a neurotrophic action, or by acting as a growth factor that was withdrawn during serum deprivation? Because virtually nothing is known about the EPOR-mediated signaling pathways in neurons, we also addressed the question whether EPO activates tyrosine kinases in postnatal rat hippocampal neurons and whether these intracellular signaling mechanisms are required for neuroprotection. The results obtained from these investigations support the hypothesis that EPO inhibits stress-induced neuronal apoptosis via activation of specific protein kinases to promote cell survival.

Methodology

Materials. MC192 hybridoma cells producing monoclonal antibody against the low-affinity nerve growth factor receptor were a kind gift from B. Hivert [Institut National de la Santé et de la Recherche Médicale (INSERM), Montpellier, France]. Brain-derived neurotrophic factor (BDNF) was a gift of Amgen.

HybernateA, Neurobasal medium, B27 supplement, and horse serum were obtained from GIBCO. Anti-nonphosphorylated-neurofilament monoclonal antibody (SMI-32) was obtained from Sternberger–Meyer (Jarrettsville, MD). R-Hu-EPO α was a gift from Ortho-Biotech (Raritan, NJ), and r-Hu-EPO β was obtained from Roche Molecular Biochemicals. One unit of EPO ≈ 10 ng protein. The kinase inhibitors PD98059 and LY294002 were obtained from Biomol (Plymouth Meeting, PA). Materials not specified were purchased from local sources.

Animal Experimentation. Procedures involving animals were conducted in conformity with the institutional guidelines that comply with national and international laws and policies.

Middle-Cerebral Artery Occlusion Model. Sprague–Dawley male rats weighing ≈250 g were subjected to MCA occlusion consisting of a small core lesion within a much larger penumbra produced by 60 min of reversible ischemia, as previously described (11). The animals received either EPO (5,000 units/kg of bodyweight) or saline i.p. at the time of occlusion. The brain was removed after 24 h, serially sectioned (50 μm thick), blocked in 3% H_2O_2 in methanol for 10 min, permeabilized for 2 min in 0.1% Triton X-100/sodium citrate at 4°C, and treated with TUNEL reaction mixture according to the manufacturer's protocol (*In Situ* Cell Death Detection Kit, Roche Diagnostics). Positive neurons were identified after development (30 min in diaminobenzidine, dehydrated, and cover slipped). As a negative control, terminal transferase was omitted.

P19 Cells. The clone P19S1801A1 was kindly provided by W. Fischer (Salk Institute, La Jolla, CA). Cells were maintained undifferentiated in DMEM supplemented with 2 mM L-glutamine/100 units/ml penicillin G/100 μg/ml streptomycin sulfate (GIBCO)/10% (vol/vol) FBS (HyClone), containing 1.2 g/liter $NaHCO_3$ and 10 mM Hepes buffer, hereafter referred to as complete medium. Serum-free medium contained the same components as above with the deletion of serum and the addition of 5 μg/ml of insulin/100 μg/ml of transferrin/20 nM progesterone/100 μM putrescine/30 nM Na_2SeO_3 (Sigma). For the experiments, 50% confluent cells were pretreated overnight with EPO or vehicle, dissociated with trypsin, washed in serum-free medium, and plated in 25-cm^2 tissue culture flasks at a final density of 10^4 cells/cm^2 in serum-free medium alone or with added EPO. Cell viability was determined by trypan blue exclusion and a hemacytometer. Nuclear morphology was studied as previously described (17). For [^{3}H]thymidine incorporation, cells were plated in 96-well plates at a final density of 10^4 cells/cm^2 in serum-free or complete medium, alone or with the indicated concentrations of EPO. After 24 h, 37 kBq/ml of [^{3}H]thymidine (Amersham Pharmacia) was added for 1 h; incorporated radioactivity was isolated by a cell harvester.

Primary Motoneurons. Spinal cords were obtained from 15-day-old Sprague–Dawley rat embryos. The ventral horn was trypsinized and centrifuged through a 4% BSA cushion for 10 min at 300 × g. Cells (representing mixed neuron/glia culture) were seeded at a density of 2,000 cells/cm^2 into 24-mm well plates precoated with poly-DL-ornithine and laminin. Motoneurons were further purified by immunopanning (18), and the cells were seeded at low density (20,000 cells/cm^2; ref. 2) onto 24-mm well plates precoated with poly-DL-ornithine and laminin, and containing complete culture medium [Neurobasal/B27 (2%)/0.5 mM L-glutamine/2% horse serum/25 μM 2-mercaptoethanol/25 μM glutamate/1% penicillin and streptomycin/1 ng/ml BDNF]. This medium (without glutamate) was readded to cultures on days 4 and 6. Cell death was induced on day 6 in culture by 48 h serum/BDNF deprivation or by incubation for 48 h with kainic acid (5 μM for mixed neuron/glia cultures; 50

μM for purified cultures). EPO (10 units/ml) or vehicle was added to the cultures 72 h before induction of cell death, and treatment continued for 48 h. The medium was then discarded and the cells fixed with 4% (vol/vol) paraformaldehyde in PBS for 40 min, permeabilized with 0.2% Triton X-100, blocked with 10% (vol/vol) FCS in PBS, incubated with antibodies against nonphosphorylated neurofilaments (SMI-32; 1:9,000) overnight, and visualized by using the avidin–biotin method with diaminobenzidine. Viability of motoneurons was assessed morphologically by counting SMI-32 positive cells across four sides of the cover slip. Staining for apoptotic bodies was done by using H33258 (17).

Hippocampal Cultures. Neurons were obtained and cultured as previously described (8, 19), and cells were resuspended in serum-free growth medium [Neurobasal A/B27 with 5 ng/ml basic fibroblast growth factor (GIBCO)/0.5 mM L-glutamine/50 units/ml of penicillin/5 g/ml of streptomycin]. For cell counting and immunofluorescence, neurons were plated on poly-D-lysine coated 12-mm cover slips in 4-well plates, at a density of 5,000 cells/cm^2. For protein extractions, cells were grown in 6-well plates coated with poly-D-lysine, at a density of 70,000 cells per well. Experiments were performed after 5 days in culture. Immediately before exposure to hypoxia, medium was removed and replaced with freshly prepared growth medium containing 0.3 units/ml EPO, or EPO in the presence of DMSO (solvent for kinase inhibitors), PD98059 (50 μM), LY294002 (100 μM), or a combination of PD98059 + LY294002 at the same concentrations. Cultures were then transferred either into a hypoxic or normoxic incubator. Hypoxia was achieved by flushing the incubator with nitrogen, which reduced the incubator oxygen concentration to <1%. Cultures were exposed to these conditions for 15 h. Cell survival was estimated under the microscope by counting trypan blue-stained neurons on 12-mm cover slips in five distinct, nonoverlapping fields of two to four different cover slips for each condition (altogether, 10–20 fields per treatment). Results are presented as means of the percentage of dead cells SD of four to five separate experiments from five different preparations. One-way ANOVA with Tukey's test (STATISTICA 4.1 for Macintosh, StatSoft, Tulsa, OK) was used to analyze the statistical differences between the groups.

Immunoblotting. Immediately after treatment, the medium was changed to another containing 0.3 units/ml EPO or 100 μg/ml BDNF and incubated at 37°C for 10 min. On ice, medium was removed, and the cells were washed twice with ice-cold PBS, lysed by adding 80 μl of 50 mM Tris·HCl (pH 7.4)/150 mM NaCl/40 mM NaF/5 mM EDTA/5 mM EGTA/1 mM sodium orthovanadate/1% Nonidet P-40/0.1% sodium deoxycholate/0.1% SDS (containing 10 μg/ml aprotinin)/1 mM phenylmethylsulfonyl fluoride. Proteins were scraped off the plate, transferred to microcentrifuge tubes onto ice (10 min) with vigorous vortexing, and finally centrifuged at 15,000 × g at 4°C for 20 min. The supernatant was mixed with 3 volumes of Laemmli buffer (250 mM Tris·HCl, pH 8.3/8% SDS/40% glycerol/20% 2-mercaptoethanol/0.04% pyronin Y), boiled for 5 min, cooled on ice, and frozen at −20°C until blotting. To assess the effects of inhibiting MAPK and/or PI(3)K activity, the cells were incubated for 30 min with DMSO, 50 μM PD98059, 100 μM LY294002, or PD98059 plus LY294002 before administration of EPO or BDNF. Protein extracts were separated by using SDS/PAGE electrophoresis (9%), transferred to a nitrocellulose membrane, and incubated with rabbit polyclonal anti-phospho-Akt/protein kinase-B (PKB; Ser-473), monoclonal anti-phospho-p44/42 MAPK (Thr-202/Tyr-204), polyclonal rabbit anti-phospho-Stat5 (Tyr-694; all from New England Biolabs), or rabbit anti-Stat5 antibodies (Santa Cruz Biotechnology). Immunoreactive bands were visualized by enhanced chemilumines-
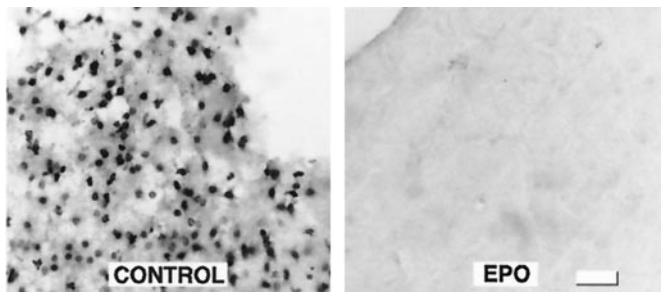
Fig. 1. EPO inhibits programmed cell death in the ischemic penumbra after middle-coronary artery (MCA) occlusion in rats. EPO administration at the onset of ischemia resulted in an almost complete protection from apoptosis (as assessed by TUNEL labeling performed 24 h later). Representative sections illustrating cells undergoing apoptosis within the ischemic penumbra in animals treated with saline (control) or r-Hu-EPO (5,000 units/kg of body weight) given i.p. at the onset of a reversible 1-h occlusion of the contralateral carotid artery. Performed six times with similar results. (Bar = 50 μm.)

Fig. 2. EPO prevents apoptosis of P19 cells. (*A*) Cells were serum-deprived for 24 h and apoptotic nuclei were counted after staining with H33258. EPO (1 unit/ml) was added as a 24-h pretreatment (EPO pre) and then removed, or added immediately after serum deprivation (EPO post). (*B*) Dose–response of the effect of a 24-h pretreatment with EPO on apoptosis induced by subsequent serum and EPO deprivation. (*C*) Time course of apoptosis induced by serum deprivation in control or 24-h EPO-pretreated cells. (*D*) Effect of EPO on thymidine incorporation in the presence or absence of serum. (Data represent mean ± SD. *, $P < 0.05$; **, $P < 0.01$ indicate significant differences as compared with serum deprivation without EPO treatment.)

cence (Amersham Pharmacia). Normalization of protein was achieved by using a pan-extracellular regulated kinase (ERK) polyclonal antibody (New England Biolabs).

Immunofluorescence Staining. After 5 days in culture, cells were washed in PBS, fixed with 4% (vol/vol) paraformaldehyde in PBS (20 min at room temperature), washed in PBS, permeabilized and blocked in 0.2% Triton X-100/PBS with 10% goat serum (20 min), and then incubated with polyclonal rabbit anti-rodent EPOR antibodies (Santa Cruz Biotechnology, 1:200 dilution) in 1% goat serum/PBS at 4°C overnight. After washes in PBS, the cover slips were incubated with Texas red-coupled goat anti-rabbit antibodies (1:100 in 1%/PBS, 60 min; Vector Laboratories), washed in PBS, dried, and mounted on cover slips with Vectashield-DAPI fluorescence mounting medium containing 4′,6-diamidino-2-phenylindole. Controls for immunofluorescence included incubation of adjacent cover slips without antibody or preadsorption of the EPOR antibody overnight with 100 times excess of control peptide (Santa Cruz Biotechnology).

Results

EPO Decreases the Number of TUNEL-Positive Neurons After Focal Cerebral Ischemia. EPO-treated animals exhibited an ≈75% reduction in infarct volume (compared with controls) 24 h after the onset of a reversible 60-min ischemia, as previously described (11). Histological examination of brain sections showed that, adjacent to a small ischemic core common to all animals, widespread tissue infarction occurred only in the saline-treated animals. This region is functionally identified as an ischemic penumbra. Brains of animals receiving EPO also exhibited a pronounced absence of inflammatory cells within the penumbra (data not shown). In sham-treated animals, numerous TUNEL-positive cells could be readily observed in marked contrast to EPO-treated animals (Fig. 1). These findings suggest that, in the middle-cerebral artery model of ischemic injury, neurons within the penumbra can be rescued from apoptosis by EPO.

EPO Pretreatment Protects P19 Cells from Apoptosis. To gain a deeper understanding of the nature of EPO-dependent neuroprotection, *in vitro* systems of apoptotic cell death were used. Apoptosis in undifferentiated P19 teratoma cells was induced by 24-h serum withdrawal, as estimated by counts of nuclei with the condensed and fragmented morphology characteristic of apoptosis after staining with the dye H33258. EPO was either administered at a concentration of 1 unit/ml 18 h before serum deprivation and then washed away, or it was added to the culture medium during the serum-deprivation period, or it was present
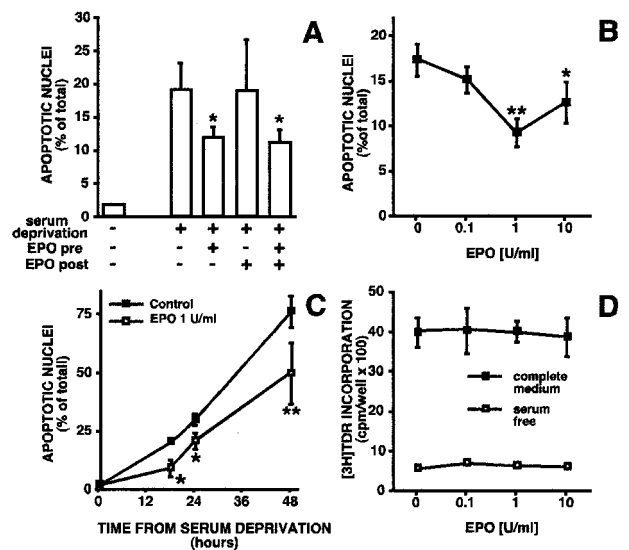
both before and during serum deprivation. Pretreatment with EPO was effective in decreasing apoptosis (Fig. 2*A*). In contrast, addition of EPO immediately after serum deprivation had no effect. A continued presence of EPO did not improve the protection conferred by pretreatment, indicating that EPO does not substitute for a serum component. The dose–response curve for EPO-mediated protection (Fig. 2*B*) indicated an optimal EPO concentration of 1 unit/ml. The improved survival of EPO-pretreated cells compared with control cells was maintained for up to 48 h after serum deprivation (Fig. 2*C*). At longer times, only very few cells in either group survived in serum-free medium. EPO-pretreated cells that survived serum deprivation for 3 days were rescued by the addition of serum, cloned, and survival was assayed again after additional serum deprivation. No differences were seen in the survival of these cells from the parental cell population (data not shown), indicating that the selection of particularly sensitive cell subpopulations does not explain the improved survival of EPO-treated cells.

EPO added to cells cultured in complete medium also improved the morphological appearance of P19 cells, producing longer and more abundant processes (data not shown). We therefore evaluated the possibility that EPO could have a proliferative effect in this model. However, this is not the case, because [³H]thymidine incorporation, either in complete medium or 24 h after serum deprivation, was unaltered by any concentration of EPO tested (Fig. 2*D*), suggesting a trophic rather than a mitogenic effect in growing cells.

EPO Is Neuroprotective and Neurotrophic in Primary Rat Motoneurons. In primary spinal cord cultures, SMI-32-positive multipolar neurons with large somata and a single long axon-like neurite are motoneurons. These cells are vulnerable to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate receptor-mediated injury (20). We used SMI-32 immunostaining to identify large motoneurons in both mixed neuron/glia culture and purified
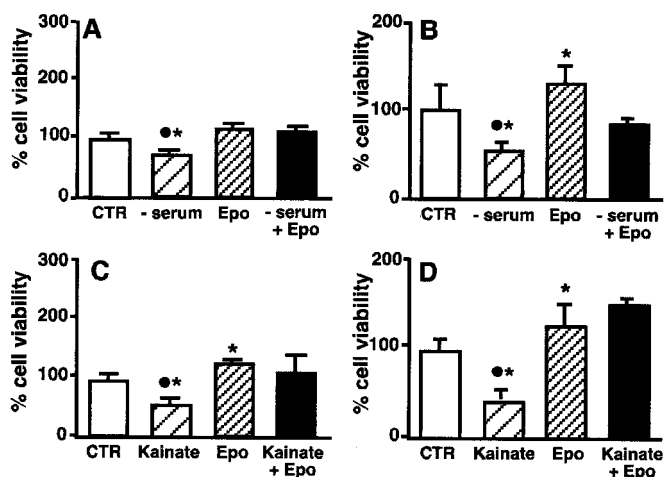
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Fig. 3. Antiapoptotic and neurotrophic effects of EPO in cultured rat motoneurons. (*A*) Cell viability in mixed neuron-glia cultures in the absence and presence of EPO (10 units/ml) in control cultures and upon serum withdrawal. (*B*) Cell viability in purified motoneuron cultures in the absence and presence of EPO (10 units/ml) in control cultures and upon serum withdrawal. (*C*) Cell viability in mixed neuron-glia cultures in the absence and presence of EPO (10 units/ml) in control cultures and upon kainate treatment. (*D*) Cell viability in purified motoneuron cultures in the absence and presence of EPO (10 units/ml) in control cultures and upon kainate administration. (Data represent mean ± SD. ★, $P < 0.05$ indicates statistical significance as compared with control; ●, $P < 0.05$ denotes statistical significance as compared with EPO during serum withdrawal or kainate treatment.)

culture conditions to observe whether EPO affects their survival under metabolic stress.

Fig. 3*A* shows the effect of 10 units/ml EPO on mixed neuron/glia cultures that were deprived of serum/BDNF for 48 h. The viability of cells, reduced by 30% in serum/BDNF-deprived cells, returned to control values (120 ± 18) in the presence of EPO, which *per se* induced an 18% increase in the number of SMI-32-positive cells. A similar protective effect was observed on purified cultures (Fig. 3*B*), indicating a direct effect of EPO on motoneurons.

EPO at a concentration of 10 units/ml was also protective against kainate-induced toxicity in this model. After a 48-h exposure to an appropriate kainate concentration, motoneuron viability was reduced by about 50% in both mixed neuron/glia (Fig. 3*C*) and purified cultures (Fig. 3*D*), whereas in the presence of EPO, the effect of kainate was abolished (Fig. 3*D*). In these experimental conditions (long-term treatment with low kainate concentration), purified cultured motoneurons stained with H33258 have a clear apoptotic morphology, displaying chromatin condensation and apoptotic bodies. The percentage of apoptotic nuclei increased from 5% in controls to 88% in kainate-treated cells. EPO reduced the number of apoptotic nuclei to 60% (data not shown).

As in P19 cells, EPO also provides neurotrophic effects in this motoneuron system. The basal survival of SMI-32-positive motoneurons in mixed neuron/glia (cultured in complete medium with serum and BDNF) was significantly better after treatment with 10 units/ml EPO for 72 h plus 48 h, as suggested by the relative percentage of cells counted after 8 days in culture (Fig. 3). Also, cellular differentiation seemed better in the presence of EPO, as indicated by the shape and size of cell soma, dendritic arborizations, and the length of axon-like processes (data not shown). The neurotrophic effect of EPO was also evident in purified motoneurons, as indicated by the quantification of SMI-32 positive cells treated with 10 units/ml EPO for 72 h plus 48 h (Fig. 3*B*).

EPO Activates MAPK and PI(3)K to Protect Hippocampal Neurons from Hypoxia. To explore EPO–EPOR signaling pathways in neurons, we used primary rat hippocampal neuronal cultures. Expression of phosphorylated forms of Stat5, Akt/PKB, and ERKs was evident in all basal samples of hippocampal neuronal cultures under normoxia (Fig. 4*A*). After exposure of neurons to 15 h of hypoxia, expression of the phosphorylated forms of these intracellular messengers was decreased, but could be largely restored by treatment with 0.3 units/ml EPO or 100 ng/ml (3.6 nM) BDNF. BDNF was used as a positive control in kinase phosphorylation studies because its neuroprotective action in cortical neurons is mediated through MAPK and PI(3)K pathways (21).

To test whether intracellular signaling through the Ras-MAPK or the PI(3)K pathway is critical for EPO effects in neurons, we used selective inhibitors of MAPK and PI(3)K to block the phosphorylation of ERKs and Akt/PKB in rat hippocampal neurons. The MAPK inhibitor, PD98059 (50 μM), completely inhibited the phosphorylation of ERK1 and ERK2, whereas treatment with the PI(3)K inhibitor, LY294002 (100 μM), resulted in the inhibition of Akt/PKB phosphorylation (Fig. 4*B*). When added in combination, PD98059 and LY294002 abolished the activation of both signaling pathways (Fig. 4*B*). The kinase inhibitors also completely antagonized the neuroprotective effect of EPO in rat hippocampal neurons upon hypoxia, when used either alone or in combination (Fig. 5). When added to normoxic cultures, neither PD98059 nor LY294002 was toxic to rat hippocampal neurons (results not shown).

To confirm EPOR expression in rat hippocampal neurons, immunostaining was performed. Cytoplasmic expression of immunoreactive EPOR in rat hippocampal neurons under normoxia was clearly detectable in neuronal cell bodies with weak staining of the processes (data not shown). Condensation of nuclear chromatin upon hypoxia was verified by counterstaining each of the EPOR-stained cover slips with 4′,6-diamidino-2-phenylindole (DAPI). EPO treatment reduced the morphological signs of cell damage. When the MAPK or PI(3)K inhibitors were used to block the neuroprotective effect of EPO, cells with nuclear condensation and fragmentation were observed (data not shown).

Discussion

The present study evaluates the nature of the neuroprotective actions of EPO. It was demonstrated *in vivo* and *in vitro* that EPO is a potent inhibitor of neuronal apoptosis induced by ischemia, hypoxia, serum withdrawal, and kainate exposure. These findings were complemented by kinase phosphorylation studies, including the use of specific inhibitors that identified which survival pathways are activated by EPO.

It is well established that antiapoptotic measures (caspase inhibitors, expression of the antiapoptotic gene *Bcl-2*) are protective in *in vivo* models of cerebral ischemia (22–24). In the present study, systemic administration of EPO abolished the appearance of TUNEL-positive cells and prevented histological damage of the penumbra after middle-cerebral artery occlusion in rats, findings consistent with the inhibition of neuronal apoptosis by EPO. Our previous study has shown that most neurons can be rescued by EPO up to 3 h (and many fewer up to 6 h) from the onset of arterial occlusion. These observations are consistent with the delay observed in the activation of apoptosis by nerve cells (25). Thus, in this model, many neurons remain viable for hours after the onset of ischemia and can be rescued by antiapoptotic therapy provided within this time window.

The antiapoptotic effect of EPO was studied in more detail in cell culture. In the P19 teratoma cell line, only pretreatment with EPO was effective in preventing apoptosis induced by serum withdrawal or kainate application. This observation suggests
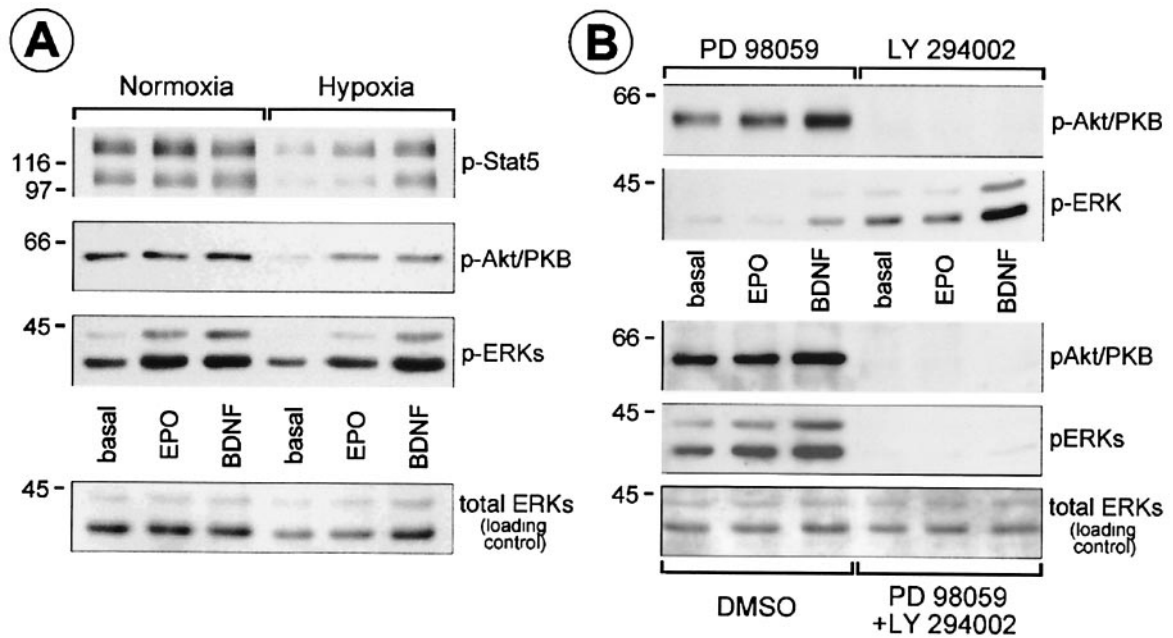
Fig. 4. EPO induces phosphorylation of tyrosine kinases in rat hippocampal neurons. (*A*) EPO (0.3 units/ml; 100 pM) and BDNF (100 ng/ml; 3.6 nM) activate Stat5, Akt/PKB, and ERKs in rat hippocampal neurons. Expression of pStat5 (lower band), pAkt/PKB, pERK1 (upper band) and pERK2 (lower band) was monitored by Western blotting. Expression of total ERKs was used as a loading control. The differences seen in total ERKs reflect variations of cell numbers in individual cultures after hypoxic conditions. An increase in the level of activating phosphorylations of Akt/PKB or ERKs relative to total protein content, however, was found to be consistent in three independent experiments. The molecular size of the protein standards are indicated (*Left*). (*B*) Inhibition by PD98059 (50 μM) and/or LY984002 (100 μM) of the EPO (0.3 units/ml) or BDNF (100 ng/ml) induced activation of Akt/PKB, ERK1 (upper band), and ERK2 (lower band) in rat hippocampal neurons upon hypoxia.

that, in this model, cells are committed to apoptosis with a much shorter latency than the *in vivo* model, or for motoneurons or hippocampal neurons *in vitro*. These neural-like cells behave similarly to other culture systems reported in which a gene expression program is initiated by EPO but requires >4 h for completion (10). The protective effect of EPO pretreatment in the P19 cell line lasted for 3 days, which is fully consistent with the induction of a gene-expression program. Further, selection of particularly sensitive cell subpopulations did not explain the improved survival of EPO-treated cells, because the survival of these cells upon a repeated apoptotic challenge did not differ from that of the parental cell population. In primary rat motoneurons, EPO increased cell viability and prevented apoptosis induced by serum/BDNF deprivation or kainate application. The cytoprotective effect was seen in both mixed neuronal/glial

Fig. 5. Inhibition by PD98059 (50 μM) and/or LY984002 (100 μM) of the EPO (0.3 units/ml) induced neuroprotection in rat hippocampal neurons upon hypoxia. ○, level of spontaneous cell death of rat hippocampal neurons under normoxia; ●, level of cell death upon 15 h of hypoxia. (Data represent mean ± SD. *, $P < 0.05$; **, $P < 0.01$ compared with hypoxia alone; n = 4–5.)

cultures as well as in purified motoneurons, indicating a direct action of EPO via neuronal EPO receptors. Also, in rat motoneurons, EPO pretreatment is still partially but significantly effective against kainate toxicity, even if it is withdrawn during the 48-h excitotoxin exposure (data not shown). When EPO was added only during the 48-h kainate exposure (without pretreatment), partial protection (70%) was still achieved (data not shown). Thus the 72-h pretreatment plus 48-h treatment conferred maximal protection.

The neuroprotective effect of EPO has been well documented in cell culture (7, 8, 10) and in rodent stroke models (6, 11–13). Our data provide evidence that distinct signaling cascades that have been characterized in hematopoietic cell lines (14) are functional in neurons and can be modulated by EPO. Even more importantly, these pathways are crucial for the neuroprotective effect of EPO, because specific inhibitors of MAPK and PI(3)K pathways largely abolished the EPO-induced protection against hypoxia-induced cell death. These signaling mechanisms have previously been shown to be involved in the antiapoptotic effect of BDNF in cortical neurons (21). Additional potential protective mechanisms that might be activated downstream from both the EPOR-Ras-MAPK and EPOR-PI(3)K-Akt–PKB interaction also include antioxidation (26), angiogenesis (27), and direct neurotrophic effects (28).

The finding that EPO not only prevents neuronal apoptosis but also has neurotrophic activity on both primary motoneurons and P19 cells extends previous studies showing that EPO provides trophic effects to cholinergic neurons (5, 29). The established role of EPO in angiogenesis (27), together with its neurotrophic action, emphasizes the regenerative potential of this molecule in addition to its neuroprotective effects.

The neuroprotective activity of EPO is not teleologically distinct from the erythropoietic one, if one considers the literature reporting an induction of EPO in the brain tissue after
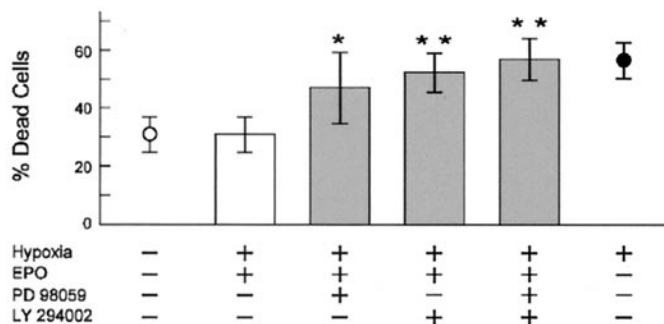
exposure to hypoxia (3, 9, 30). EPO production by hypoxia occurs through hypoxia-inducible factor 1 (HIF-1), which also induces expression of several glycolytic enzymes to reorient energy metabolism toward favoring survival during hypoxia (31). Increased kidney production of EPO represents the main adaptive mechanism to hypoxia by the organism through augmenting the number of erythrocytes and, thus, tissue oxygenation. At the same time, EPO protects neurons, the cells most sensitive to hypoxic damage. It is important to note that EPO production, via HIF-1, is induced not only by hypoxia but also by hypoglycemia and oxidative stress (32, 33). These endogenous protective mechanisms are clearly insufficient upon acute injury, as demonstrated by the significant effect of exogenously administered EPO *in vivo*. It is possible that the brain does not produce enough EPO after cerebral ischemia, or that the latency of neosynthesis is too long to sufficiently protect neural tissue. The inflammatory component of cerebral ischemia may, in fact, limit EPO production; as tumor necrosis factor, whose production in ischemic brain has been demonstrated (34), directly inhibits EPO production *in vivo* (35).

In vivo experiments (11) suggest that inhibition of neuronal apoptosis by EPO is not its only protective action, as EPO also decreases the inflammatory infiltrate induced by cortical trauma in mice and ameliorates experimental autoimmune encephalitis. These effects could arise via a direct role of EPO as an antiinflammatory cytokine, or they could be secondary to protection from neuronal apoptosis. Although apoptosis has generally been regarded as a noninflammatory mechanism of cell death that, in contrast to necrosis, does not involve a breakdown of cell membrane and release of intracellular components, the hypothesis that inflammation might be secondary to apoptosis has been suggested recently. Blocking apoptosis by caspase inhibitors prevents inflammation and induction of inflammatory cytokines and has protective effects in a rat model of cerebral (36) or renal (37) ischemia. A direct anti-inflammatory effect of EPO at the level of the cytokine network cannot be excluded, because it was reported that EPO decreases *ex vivo* production of tumor necrosis factor and augments production of IL-10 in blood cell cultures from hemodialysis patients (38). Finally, reperfusion hyperexcitability could also be reduced, because EPO seems to affect directly neuronal excitability; we have observed that it elevates the seizure threshold for the convulsant kainate (11). In this manner, EPO may also limit neuronal necrosis.

In conclusion, our data show that EPO prevents neuronal apoptosis induced by oxygen, nutrient, growth factor deprivation, or kainate exposure. The specific pathways responsible include activation of extracellular signal-regulated kinases and phosphotidylinositol 3-kinase, counteracting hypoxia-induced down-regulation of these pathways, similar to those observed for BDNF. The biological difference here is that in addition to its local autocrine–paracrine effects, EPO is also a circulating hormone. Thus, the potential exists for cross-talk to occur between the systemic and the local systems.

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